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Hematology of Healthy and Virus-diseased Sockeye Salmon, *Onchorhynchus nerka*[1]

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(Plates I-III; Text-figures 1-4)

Introduction

Epizootiological investigations have been routine in this laboratory[4] ever since a virus disease began depleting the Federal hatchery stocks of fingerling sockeye salmon in 1951 (Rucker *et al.*, 1953; Watson, 1954; Watson, Guenther & Rucker, 1954). Only cursory observation of stained blood films had been made from diseased fish. More detailed hematological studies were instituted by a short term budget allotment to determine the characteristic blood picture of the virus disease. It was also of interest to find a rapid diagnosis of the disease from either live or stained blood films. In addition, determination of hematocrits was undertaken.

Literature Review

Hematology of teleosts is still a pioneering field of research. Reports on morphology of normal fish blood cells are not scarce but are contradictory. The best reviews of this subject are found in the doctoral dissertations of Yokoyama (1947) and Katz (1949). Dombrowski (1953) has described the normal blood picture of 15 species of fresh water fish.

The very scant literature on hematological characteristics of diseased fish is described by Katz (1950). The hematology of carp afflicted with stomach dropsy has been discussed by Lyman & Shpolanskaia (1949), Schäperclaus (1953) and Dombrowski (1954). Glucksman & Gordon (1953) have studied the hematology of normal and melanomatous fish.

No reports have been found on the observation of blood cells of fish with a phase-contrast microscope although exploratory and also significant hematological studies of higher vertebrates have been undertaken with this equipment (Bennett *et al.*, 1951, p. 213).

Values for percent. hematocrit (the volume of packed red blood cells) have been found for 10 species of fresh water fish and 4 species of marine fish (Young, 1949; Aserinsky, 1954; Lepkovsky, 1930; Hall, Gray & Lepkovsky, 1924; Wilson & Adolph, 1917; Vars, 1934; Field, Elvehjem & Juday, 1943; Benditt, Morrison & Irving, 1941). The average hematocrit values for fresh water fish varied from 24.8 to 46.1 percent.; for marine fish from 13.5 to 29.5 percent. Methods used for obtaining blood were cardiac or bulbous puncture, severance of tail and pricking of caudal artery above the posterior margin of the anal fin.

It is believed that this study will report the first account of a micro-hematocrit method for small fish (30-60 mm. in fork length). These fish could deliver only one or two drops of blood after their tails had been severed.

Materials and Methods

The sockeye salmon fingerlings used in this study were reared at the Federal hatchery in Leavenworth, Washington, 130 miles from Seattle, Washington, and were trucked to the laboratory for use in experimental infections. In Seattle, 200 of these fish were kept in running spring water in a baked enamel trough, 4'×1'× 6", held at 50-54° F. The water level averaged 5 ½". Sockeye fingerlings which contracted the virus disease by natural infection were kept at the Leavenworth hatchery in a trough which

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contained 13,000 fish at the onset of the disease. These diseased fish were brought to Seattle in lots of 400 for three successive weekends. At Seattle hematological studies were made on fish naturally infected with the virus disease and on fish which had earlier been experimentally exposed to the virus.

Experimental Procedures for the Study of the Virus Disease

Preparation of virus material for experimental infection of fish.—The original source of the virus for this research was virus-diseased fish naturally infected in hatcheries in the state of Washington. This virus material was passed through at least four serial transfers prior to the making of a frozen stock suspension. The suspension consisted of a frozen (—40° C.) emulsion of infected fish which had been diluted 1:3 with tap water and sealed in glass vials. After six months, this culture was again transmitted through 13 serial transfers before use in the experiment. For this study, fish from the 13th serial transfer were blended in an "Osterizer" for 30 seconds and centrifuged for 3 minutes at approximately 2,000 rpm. The supernatant was then filtered through a 7 lb. Mandler filter. The filtrate was diluted with sterile tap water to a concentration of 1:100. For the artificial infection, 200 fish were suspended for 15 minutes in a gallon of Mandler filtered suspension diluted 1:100. Control fish were given the same treatment as the infected fish except for the exposure to the virus agent.

Protocol.—Antecedent to the experiments recorded in this study, four trial infection experiments were carried out in order that hematological characteristics of the virus disease could be determined. Following these, three lots of 200 fish each were used. One lot comprised the control or uninfected fish. On May 11, 1954, the two remaining lots were suspended in 1:100 dilution of infected material for 15 minutes. Beginning on May 12 and daily thereafter through May 20, then again on May 23 and June 7, the following techniques were practised. An average of 6 infected fish and 6 control fish were used daily, and for each a Wright-stained and a peroxidase-stained blood film was prepared. One live film was studied with a phase-contrast microscope, either unstained or supra-vitally stained with brilliant cresyl blue, and one hematocrit was made. Lack of blood or time sometimes prevented observation of a live film on all 12 fish. Blood samples from 73 infected and 61 control fish were analyzed during the experiment.

On May 15, naturally infected fish were brought into the Seattle laboratory from the virus-infected trough at the Leavenworth hatchery. The protocol described above for the experimentally infected fish was followed with these naturally infected fish on May 16, 21, 22, 23, 29, 30, June 1 and 9. Controls were likewise employed with these fish. Sixty-four infected and 28 control fish were studied.

Preparation of equipment and fish for hemic studies.—Microscope slides and coverslips were soaked in concentrated nitric acid for a minimum of 4 hours, then washed in running tap water for 4 or more hours, then rinsed in distilled water and finally stored in 95 percent. ethyl alcohol. Slides and coverslips were dried with clean gauze just prior to use.

Fork length, head length, general external appearance and activity were recorded for each fish. The fish was then placed between blotters or covered with gauze in such a way that only the portion of the body immediately posterior to the anus was exposed. This exposed portion was dipped into 95 percent. ethyl alcohol, dried and wiped quickly with gauze. The tail was then severed from the body with scissors by a quick firm snip in the region immediately posterior to the anal and adipose fins. The drop of blood which oozed from the severed caudal vein and artery was touched to a capillary hematocrit tube. After sufficient blood had entered the tube, the remainder of the drop of blood on the fish was first touched to one coverslip from which a stained film would be made and then touched to a second coverslip from which a live wet film or a supra-vitally stained film would be prepared. For these small fish, cover slips used were 15 mm. square, No. 1 thickness.

Stained blood films.—All blood films were routinely prepared by the standard coverslip technique (Wintrobe, 1949). One coverslip of the pair used was stained with Wright's stain and the other with peroxidase stain (Hughes, 1953). The freshly prepared and dried films were not fixed in methyl alcohol prior to staining. The peroxidase stain was made with absolute methyl alcohol instead of 95 percent. alcohol, hence fixation was unnecessary.

Live unstained and supra-vitally stained blood films.—A freshly cleaned slide was lowered carefully toward a drop of blood on a coverslip until the blood touched the slide. The slide was then upturned and the edges of the coverslip ringed with petroleum jelly. For supra-vital staining, a freshly cleaned coverslip was touched by a very small drop of brilliant cresyl blue (0.1 percent. in absolute ethyl alcohol, freshly filtered). A second clean coverslip was placed over the slip containing the stain, and the two were pulled apart immediately. As soon as the stain on each coverslip dried (a few seconds),

the stained coverslip was ready to receive a drop of blood.

Micro-hematocrits.—A self-filling capillary tube[5] with a capacity of 5 or 10 lambda or the narrow stem of a red blood cell diluting pipette was used as a hematocrit tube. After a tube was filled with blood, the length of the column of blood was measured with a micrometer calipers which was read to tenths of a millimeter. The end of the tube at which the blood had been introduced was closed with sealing wax. The hematocrit tube was inserted into a piece of thick-walled rubber tubing before it was placed in a centrifuge, thus cushioning it from damage by the sides of the metal centrifuge tube holder.

All samples were centrifuged in a clinical model centrifuge at 3,000 rpm. for 35 minutes, and micrometer measurements of red blood cell volume and total volume were recorded within half an hour after centrifuging. If the volume of blood recorded before centrifuging varied more than 1 mm. from the volume measurement of total blood after centrifuging, the sample was discarded.

The use of an anticoagulant was necessary. The hematocrit tubes were rinsed in a solution of heparin (0.25 mg./cc. of sterile distilled water) and blown dry with a jet of air, rinsed a second time with a fresh drop of heparin solution and again blown dry.

Hematocrit tubes were cleaned first by ejecting the wax plug with a fine wire and then by rinsing several times in normal saline and 95 percent. ethyl alcohol. Occasionally a rinse in concentrated nitric acid was required to clean the tube thoroughly.

Phase-contrast microscopy photographs.—An American Optical phase-contrast microscope was used with an American Optical (No. 735) spherical microscope illuminator or a Wilcox Strobolite with or without ground glass interference. A 1.8 mm. medium dark-contrast objective was used. The camera was a 4×5 Speed Graphic adapted for photomicrography.

Morphology of Normal Blood Cells

Time permitted only a few blood counts on normal and diseased fish. Hence, the following account can only be qualitative. Normal, apparently healthy fingerlings provide a very uniform blood film for study. Mature erythrocytes are so numerous that one must examine the blood film carefully to detect the presence of the few lymphocytes and clusters of thrombocytes. Occasionally a neutrophil is seen as the only representative of a granulocyte.

[5] Obtained from Wakefield Industries Corporation, Skokie, Ill.

One to 2 percent. of the erythrocytes are orthochromatophil or polychromatophil normoblasts. They can be distinguished from the mature erythrocytes by their chromatin pattern, characteristics of the mitochondria and the size of the nucleus relative to the size of the cytoplasm.

In the nucleus of a mature erythrocyte (Plate I, Fig. 4, left of center), the nuclear membrane is very thin, the chromatin particles are small and form a tightly meshed network. Two examples of an orthochromatophil normoblast seen in Plate III, Fig. 13, show that the nuclear membrane is more distinct than that in a mature erythrocyte and that the chromatin particles are slightly larger and less tightly meshed. In a polychromatophil normoblast (3 cells in Plate III, Fig. 14), the chromatin particles are large and form a coarse-stranded meshwork; the nuclear membrane is definitely thicker than that seen in the mature erythrocytes.

Mitochondria in a mature erythrocyte are few in number (8-12) and appear as tiny dots or short rods. In an orthochromatophil normoblast the number of mitochondria is roughly twice that found in a mature erythrocyte, and the majority of them are short, fat rods, although a few may be long, thin rods. In a polychromatophil normoblast, the majority of the mitochondria are long, very thin rods which sometimes appear tangled or interwined. The cytoplasm of the polychromatophil normoblast is crowded with mitochondria. In a live blood film, stained supra-vitally with brilliant cresyl blue, the mitochondria in all erythrocytes become a light shade of blue when examined under bright-field microscopy.

The cytoplasmic area in a polychromatophil normoblast is less than that in either an orthochromatophil normoblast or a mature erythrocyte. The cytoplasm of a mature erythrocyte appears to cover a large surface area because the nucleus is slightly smaller than that of the polychromatophil or orthochromatophil normoblast. The nucleus in both the mature erythrocyte and in the orthochromatophil normoblast is oval, while that in the polychromatophil normoblast is usually more round than oval.

The size of the 3 erythrocytes just described is variable and is directly related to the proximity of either an impending or recent mitotic division. Cells which have recently divided can be as small as 17 microns on their long axis. Cells near division can be as long as 22 microns. The average dimensions of the majority of mature erythrocytes are 19×11 microns. The average nucleus measures from 7-9×4-6 microns.

A basophil normoblast, rarely seen in the

blood films of healthy fish, is labeled *BN* in Plate I, Fig. 4. The nucleus and cytoplasm have almost the same index of refraction, and the nuclear chromatin appears homogenized but does contain indistinct areas of chromatin agglomerations. A thin, but definite, nuclear membrane is present, and occasionally the chromatin forms small thickenings on the inner surface of the nuclear membrane. Mitochondria are long and so thin that they are difficult to discern. Occasionally the mitochondria are arranged like small beads on a string instead of being thin, smooth rods.

The leukocytes seen in normal sockeye salmon fingerlings are lymphocytes, thrombocytes (the nucleated homologue of the human platelet) and neutrophils (heterophils). A small active lymphocyte, labeled *L,* is shown in Plate III, Fig. 15, and a small quiescent lymphocyte occupies the center of Plate III, Fig. 16. The active lymphocyte has a round nucleus which occupies almost the entire cell, and that part of the cytoplasm which is visible forms a pseudopod containing short rod-like or small dot-like mitochondria. In Plate III, Fig. 16, the mitochondria point inward toward the indented part of the nucleus. These mitochondria could not be resolved photographically. An example of a large lymphocyte, which is seldom seen in healthy fish, is pictured at the lower right of Plate II, Fig. 9. The mitochondria in this large lymphocyte are larger than those in the small lymphocyte and form either long rods or spindles. In both large and small lymphocytes, the nuclear membrane is etched sharply. The chromatin in the small lymphocyte is coarsely clumped, and the open meshwork between chromatin strands is refractile (Plate III, Fig. 16). The chromatin in the large lymphocyte consists of coarsely meshed strands, but the open meshwork is less refractile than that in the small lymphocyte. Neither the small lymphocytes (5 to 10 microns in diameter) nor the large lymphocytes (18 to 25 microns in diameter) display movement other than a very slow oozing or rearrangement of the cytoplasm.

Small lymphocytes and thrombocytes appear quite similar when a live blood film is examined for the first minute after its preparation. Following the first minute, the thrombocytes bleb or balloon out, thus performing their natural function of clotting. A blebbed thrombocyte occupies the right center of Plate I, Fig. 1. The white circular dot near the upper border of the thrombocyte is seen only in virus-diseased fish. The fine threads of fibrin can be seen radiating from the periphery of the cell. An unblebbed thrombocyte in a fingerling sockeye salmon would resemble the lymphocyte pictured in Plate III, Fig. 16, except that the nucleus of the cell would have only a small crevice instead of a deep indentation or the nucleus would completely fill the cell. Only 3 or 4 tiny round granules may be seen in the cytoplasm of a thrombocyte. As a fingerling sockeye salmon approaches a length of 50 mm., a few mature thrombocytes can be seen. These are similar in appearance to the size and shape of the nucleus in a mature red blood cell. A thin, almost imperceptible, rim of cytoplasm encircles the nucleus. In live or stained films from healthy fish, the thrombocytes are usually clumped in groups of 2, 3 or 4.

The most motile cell in a live blood film is the neutrophil or heterophil. As a rule the nucleus trails while the cytoplasm leads the direction of motion. A nonfilamented neutrophil is pictured at the center right of Plate I, Fig. 3. The white refractile dot in the cytoplasm is only characteristic of neutrophils in fish afflicted with the virus disease. This non-filament neutrophil is considered a metamyelocyte which can be recognized by the appearance of the nucleus in an active cell; the nucleus alternately has a hump (as in Plate I, Fig. 3) or a depression midway along its length, as if it performed an undulating motion. The cytoplasm also contains a few tiny round granules which appear slightly darker than the mitochondria. Metamyelocytes are not common in the blood of healthy fish.

The bi-lobed neutrophil is normally the predominant stage seen in the peripheral blood, although highly polymorphic nuclei with 3, 4 and 5 lobes are also occasionally seen. No promyelocytes or myelocytes could be identified as such in any live films examined. After the peroxidase test, the cytoplasm of all neutrophils, regardless of their maturity, was replete with purplish black granules.

Basophils and eosinophils are easily identifiable with phase-contrast microscopy. The eosinophil is a small round cell (8-12 microns) which is entirely filled with minute refractile granules (1-2 microns). These granules are as luminous as a mass of tiny, very bright, light bulbs. The nucleus was rarely seen. When visible it was thin and flattened at one edge of the cell membrane. An eosinophil has never been seen in motion.

Though eosinophils and basophils are uncommon in healthy fish, from 5 to 10 of each have been seen on a single blood film from starved fish, virus-diseased fish and fish infected with bacterial gill disease. Plate II, Fig. 8, depicts one type of basophil which is the same size and shape as the eosinophil just described. Instead of very refractile granules, the basophils have very dark granules which are encircled by a bright halo when viewed under a phase-contrast micro-

scope. The nucleus of the basophil in Fig. 8 is not visible. All of the granules, which really fill the entire cell, are impossible to focus in the same plane; thus the pictured cell appears to be only partially filled with granules. On the other hand, the majority of the granules filling the cytoplasm are visible in the basophil pictured in Plate II, Fig. 9. This cell is flattened, while the basophil in Fig. 8 is rounded. In a flattened basophil, the nucleus is eccentric and has a coarse network of chromatin which is disposed in thick clumps at points of anastomoses. No instance of a polymorphic nucleus has ever been seen in live or stained films. The basophils in Figs. 8 and 9 showed no signs of movement; however, a third type of basophil illustrated in Plate II, Fig. 7, glided slowly with the cytoplasm trailing the nucleus. This is an immature cell, there being fewer and smaller cytoplasmic granules. The diffuse chromatin reveals a faint network.

The granules in all three types of basophils just described have in common an unusual internal characteristic. Each granule contains 2 or 3 parallel striae which appear darker than the granule itself. In addition these granules are specifically stained a deep bluish-purple by the supra-vital dye, brilliant cresyl blue. No other cellular component in the blood of sockeye salmon is so colored by this stain. When one is searching for the presence of basophils in a supra-vitally stained blood film, they can be easily detected by their purplish color during examination with bright-field microscopy and 100 magnification. Corroborating evidence for the identification of basophils of teleosts by their supra-vitally stained granules may be found in Michels (1938, p. 327).

Morphology of Blood Cells Associated with the Virus Disease

When viewing either a stained or live blood film from a fish heavily infected with the virus disease, one is immediately impressed with the tremendous amount of cellular debris and unidentifiable particles present in almost every field of observation. From a study of a stained film alone, it would have been impossible to determine the origin of this debris, examples of which are shown in Plate I, Figs. 2, 4 and 5, and Plate II, Figs. 10 and 11.

Besides the cellular debris in the blood, the next most obvious characteristic is the delayed clotting time. Ordinarily, with healthy fish, one must work with utmost speed when preparing live or stained blood films in order to prevent clotting, which occurs in about 15 or 20 seconds. Blood from a virus-diseased fish will remain fluid as long as 3 minutes after exposure to air.

At the first sign of decreased clotting time, the thrombocytes in a Wright-stained blood film have a smudged, unevenly faded blue appearance, instead of the normal, homogeneously dark purple. They may or may not have a tiny, dark brown, round pigment spot in their cytoplasm. This pigment spot is about 1 micron in diameter when it is round, but it also may appear as a short fat rod (2×1 microns). With phase microscopy, this included pigment spot appears as a refractile dot. The thrombocyte in Plate I, Fig. 1, is in its earliest stage of degeneration and possesses an intracytoplasmic refractile dot. Plate I, Fig. 2, shows a totally degenerated thrombocyte with 3 pairs of pigment granules. If a live film is examined with bright-field microscopy, these refractile dots appear dark brown to black. These pigment granules have no affinities for stain in a Wright-stained film but do retain the dark brown color which is seen in a live film under bright-field observation.

A totally degenerated thrombocyte, as seen in Plate I, Fig. 2, with phase-contrast microscopy in a live mount, cannot be recognized as such in a Wright-stained film. One can only see the pigment granules in a stained film with bright-field microscopy; however, if the stained film is examined with a phase-contrast microscope, the outline of the degenerated thrombocyte with its included pigment granules is made visible. Variously diseased salmonoids other than sockeyes have been examined and no live or stained blood film has disclosed degenerated or degenerating thrombocytes with included pigment granules. Pigment granules free in the serum are occasionally seen in healthy fish but their presence has been attributed to leaching of pigment from the incised tissues of the tail region during the preparation of the blood film.

During one phase of the virus disease there exists a true basophilia which reveals the existence of the three types of basophils described earlier and pictured in Plate II, Figs. 7, 8 and 9. The majority of the basophils are of the type seen in Fig. 9. Gradually the large numbers of normal-appearing basophils are replaced by degenerating basophils, an example of which is illustrated by Plate II, Fig. 10. The degenerating basophil usually does not have pigment granules, but in a few instances they have been seen. Fig. 10 indicates the pigment granule as a white circle with a gray center dot. The granules in a degenerated basophil are irregular in shape rather than round and do not take a brilliant bluish-purple stain but rather a faded blue color in a film supra-vitally stained with brilliant cresyl blue. The fine striae seen in the granules of normal basophils no longer exist in the irregularly-shaped granules of the degenerating basophil.

Another characteristic of the blood picture in a virus-diseased fish is clasmatosis—the dissemination or scattering of basophil granules, and to a far lesser degree, the dissemination of eosinophil granules in the blood stream. The extracellular granules of basophils are usually enclosed in bits of cytoplasm and appear singly, paired or as many as four in a group (Plate II, Fig.11). In a Wright-stained film there have been only single, isolated instances when these extracellular granules have been located adjacent to or in the immediate vicinity of mature basophils. It is not believed that the granules represent an artifact produced by the mechanical rupture of the basophil. In a live film, they do not possess the fine striae which exist in the intracellular granules. Also, these extracellular granules take on either no color or only a faded blue color in a blood film supra-vitally stained with brilliant cresyl blue. Macrophages have been observed with faded blue granules recently included in their cytoplasm (Plate I, Fig. 6) Phagocytized eosinophil granules have not been observed.

After the peak of mortalities has passed, engorged and degenerating macrophages outnumber all other leukocytes. The nuclear chromatin forms a diffuse spongy network; the cytoplasm has long thin mitochondria and a few dark round granules (Fig. 6) Macrophages are monstrously large cells—one occupying the space which three normal erythrocytes would fill if placed side by side. Little or no ameboid movement was witnessed in macrophages. As many as 12 pigment granules have been seen in an engorged macrophage. Plate I, Fig. 5, shows a macrophage which had recently ingested a large degenerated lymphocyte. The nucleus of this macrophage is a thin lunate structure pushed toward the periphery of the cell.

The presence of many large lymphocytes is also characteristic of the blood of fish severely infected by the virus (Plate II, Fig. 9, center right). Degeneration in the large lymphocyte is manifested by an included pigment granule or cytoplasmic vacuolization, or both. The vacuoles are not refractile. In a Wright-stained film, a degenerated lymphocyte has a thin rim of pale blue cytoplasm and a gray to grayish-blue nucleus. A large normal lymphocyte has a reddish-purple nucleus.

The large leukocyte seen in Plate II, Fig. 12, is as yet unidentifiable. It has rarely been found in the blood of healthy fish. Its cytoplasm differs from that of all other leukocytes in that it contains only small spherical granules which are dark and do not resemble the mitochondria seen in other leukocytes. These granules are about one-third the size of those found in a basophil. Some overlie the nucleus, which appears as a diffuse mass of chromatin circumscribed by a thin membrane. The cell has a slow creeping motion with the cytoplasm leading the nucleus. No counterpart for this cell in a stained film could be found. Further study on larger numbers of these cells is necessary before a definite identification can be made. At this time it will be referred to as a monocyte.

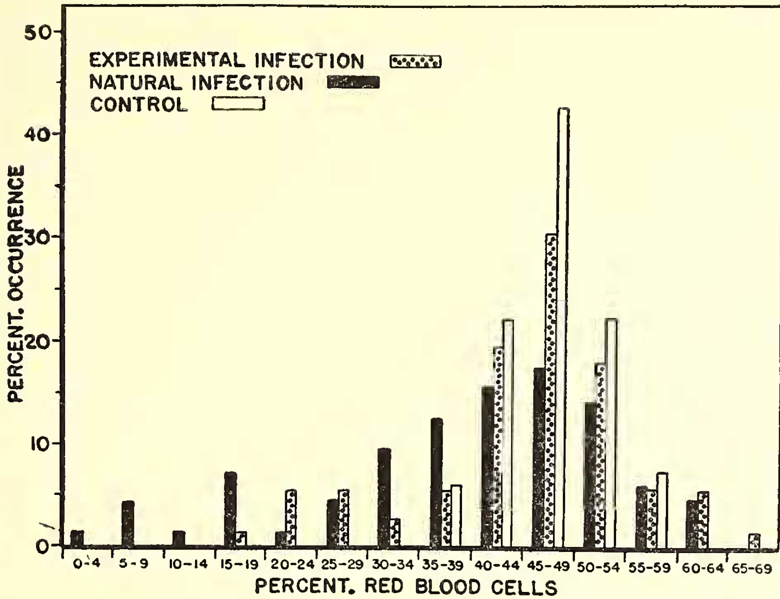
In fish which are recovering from the virus disease there is an extremely high increase in the number of immature erythrocytes (basophil normoblasts particularly). Many of the immature erythrocytes are undergoing mitosis, which is rarely seen in the blood of healthy fish.

Hematocrits

Healthy fingerling sockeye salmon.—The average percent. hematocrit of 68 control fish was 47, representing a range of 35.8 to 57.9 and a variation of 38 percent. (Text-fig. 1). The fish whose hematocrits were in the range of 35 to 39 and 55 to 59 percent. (8 fish) did not present a truly healthy blood picture upon analysis of their Wright-stained films. A variety of abnormalities was represented, neutrophilia and anemia being the most prevalent. They did not show any evidence of the virus disease.

The hematocrits of the control fish used in conjunction with the experimentally virus-infected fish are plotted in the upper part of Text-fig. 2. Beginning the seventh day after artificial infection, the controls also showed evidence of infection not only from the analysis of the live and stained blood films but also from the rise of the average percent. hematocrit. It is not possible to ascertain how the accidental contamination of the control trough occurred. Both the controls and the infected fish were kept in the same hatchery; however, separate equipment was assigned for each trough. The main stock of healthy fish, from which all experimental fish were obtained, was kept in a different hatchery. These stock controls did not acquire the virus disease; hence, accidental contamination was presumed to have occurred in the controls used. A new group of control fish was selected from the stock of healthy controls for obtaining the data listed under the twelfth and twenty-seventh day after infection.

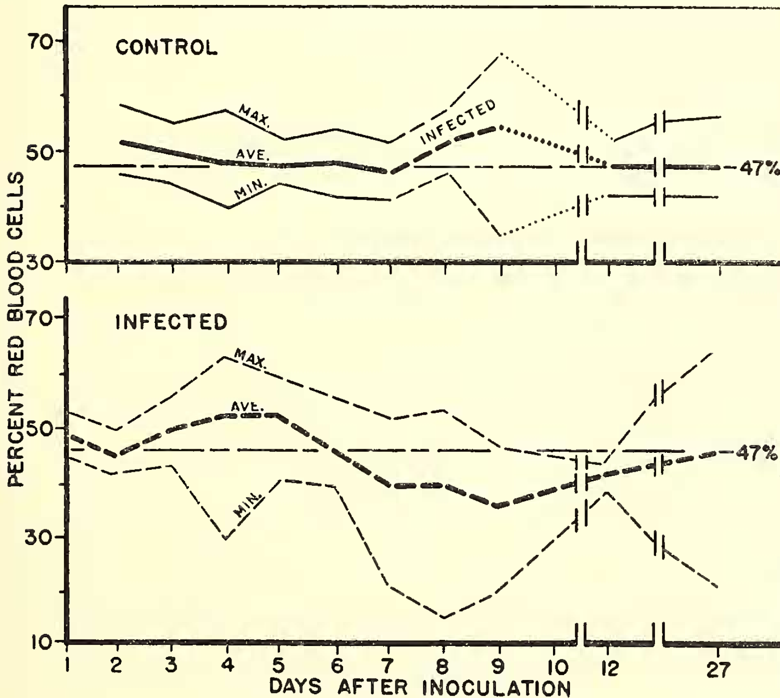
Fingerlings experimentally infected with the virus.—The average peak hematocrit was 54.8 (maximum 68) for the accidentally infected controls and 53 (maximum 64) for the experimentally infected fish. For the latter group this peak occurred on the fourth day after infection. From Text-fig. 3 it is possible to correlate the average hematocrit with the percentage of daily mortality. Two days following the peak hematocrit, the maximum number of mortalities oc-



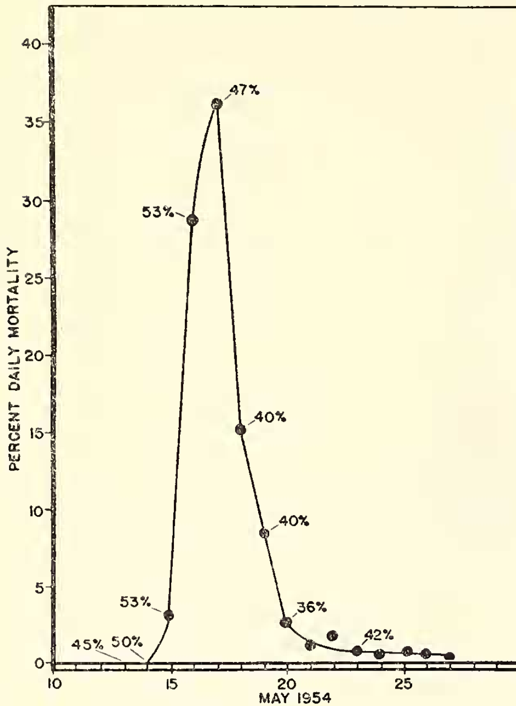
TEXT-FIG. 1. Hematocrit values of fingerling sockeye salmon – control and virus-diseased.

curred—35 percent. of the total fish inoculated with the virus. The explanation for this may be that, because of the shock incurred from the infection, there is a loss of plasma which accounts for the rise of average percent. hematocrit; following the effects of shock, the plasma level and the normal percent. hematocrit (47) are restored in the surviving fish by the sixth day after exposure to the virus.

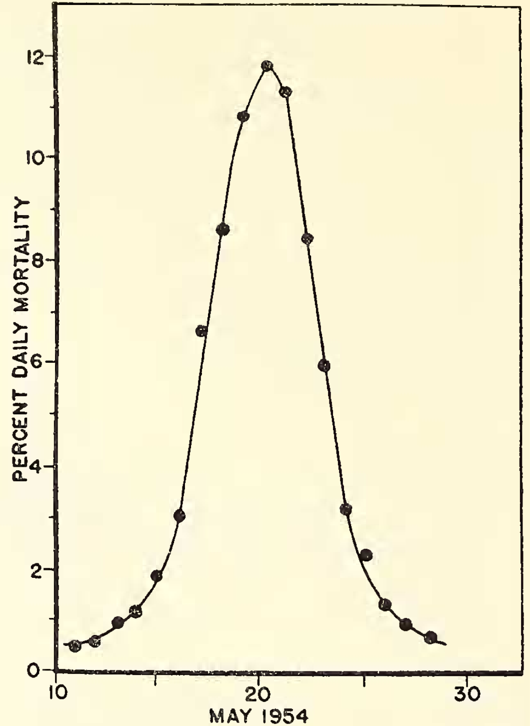
Fingerlings naturally infected with the virus.— On May 12, 1954, only 4 fish survived transport to Seattle from the disease-infected trough at the Leavenworth hatchery. It was possible by the examination of live blood films of these fish with a phase-contrast microscope to detect the typical blood cells associated with the virus disease heretofore studied only in experimental infections. The next lots of diseased fish were trans-



TEXT-FIG. 2. Maximum, minimum and average hematocrit values of control and experimentally virus-infected fingerling sockeye salmon.



Text-fig. 3. Percentage of daily mortality of fingerling sockeye salmon experimentally infected with the virus disease, and average hematocrit values of moribund fish delineated on the mortality curve.



Text-fig. 4. Percentage of daily mortality for the duration of the virus infection in a trough containing 13,000 fingerling sockeye salmon naturally infected at the hatchery in Leavenworth, Kansas.

ported to Seattle on May 16, 21 and 28. Since an insufficient number of samples was studied from the onset of infection up to the day of peak mortalities (May 11-May 20), complete hematological data for the entire course of the disease could not be tabulated as was done for the experimentally infected fish (Text-fig. 2).

From May 1 to May 10 the daily mortalities in the diseased trough at Leavenworth ranged from 8 to 24 fish a day. On May 11 there were 65 dead and on May 12 there were 89. A cumulative mortality of 50 percent (6,500 fish) was recorded on May 20, on which day also occurred the peak mortality (1,620 fish) (Text-fig. 4). By May 28 the cumulative mortality had reached 80 percent. These mortalities were comparable both in total magnitude and daily incidence to those which had occurred during the virus epidemic at this same hatchery the preceding year (Watson, Guenther & Rucker, 1954).

The highest hematocrit recorded for the naturally infected fish was 64 percent. on May 21. The lowest hematocrits were 16 percent. for the experimentally infected fish and 1 percent. for the naturally infected fish. Beginning May 22 and continuing through June 7, hematocrits below 35 percent. were recorded for 30 percent. of the fish examined. By contrast, only 15 percent. of the experimentally infected fish registered hematocrits below 35 percent. Infected fish in both groups registering hematocrits below 35 percent. displayed erythema at one or more of the following areas: base of any or all of the fins, at the anus and the isthmus. These low hematocrit values were probably caused not only by the erythema but also by the necrosis seen in hemapoietic areas of kidney tissue sections.[6]

Microscopic findings related to the hematocritis.—The hematological schedule on page 35 was compiled, presupposing one single fish could have been bled every day from the onset of exposure to the virus infection. These data are based on the results obtained from both experimentally and naturally infected fish.

Discussion

This study has demonstrated that phase microscopy provides more useful visibility of live blood cells than does bright-field microscopy of stained blood cells. During the course of the virus disease, certain blood cells were difficult to differentiate with certainty by their staining characteristics because they were undergoing de-

Days after Infection	Percent. Hematocrit	Microscopic Blood Findings
1	45-50	No change from the normal.
2 3	45-50 50-55	A few thrombocytes possess one pigment spot; increase in number of metamyelocyte neutrophils.
4	55-65	Majority of thrombocytes and neutrophils contain one to four pigment spots; delayed clotting time; a few basophils; first mortalities occur.
5 6	65-55 55-45	Degenerated thrombocytes and neutrophils; a large number of basophils (10-12 on each blood film); a few large lymphoctyes, eosinophils and macrophages; extracellular granules of basophils; by the end of the sixth day maximum daily mortalities occur.
7	45-35	Large number of macrophages; pigmented degenerated thrombocytes, neutrophils; basophils and large lymphocytes; extracellular granules of basophils.
8 9	35-10 10-30	Immature erythrocytes outnumber mature erythrocytes; mitotic division seen in many of immature erythrocytes; macrophages; many unidentifiable particles of degenerated cells; clotting time still prolonged.
12	30-40	Polychromatophil and orthochromatophil normoblasts in minority, no basophil normoblasts; a few normal neutrophils and thrombocytes.
27	40-50	Clotting time normal; blood picture normal; first evidence of scoliosis in recovered fish.

[6] Unpublished data.

generation or were completely degenerated. The possible vagaries and variations in stains and staining techniques make it difficult to reproduce results. The characteristic striae seen on each basophil granule were not seen in a stained blood film, nor were they reproducible photographically. Thus, they could only be observed by phase microscopy.

Basophilia has not been recorded in published reports as a diagnostic feature of any other fish disease. The hematology of stomach dropsy in carp has been described in two separate studies. Dombrowski (1954) reported a decrease in lymphocytes and an increase in leukocytes, followed by degeneration of the leukocytes (a term which Dombrowski used without qualification). Lyman & Shpolanskaia (1949) also reported a decrease in lymphocytes, but were specific in naming the types of increased leukocytes, although they did not describe them. At the severest stage of infection, monocytes increased from the normal 5.7 to 38 percent., neutrophils from 0.2 to 7.0 percent., polymorphonuclear cells from 2.1 to 11.2 percent. The type of cells included in the term *polymorphonuclear cells* was not stated. Eosinophils were not found in the peripheral blood until the third year; the term *basophil* was not mentioned.

Besides basophilia, other notable characteristics of the virus disease are prolonged clotting time, the existence of pigment particles in the degenerating thrombocytes and neutrophils, and extracellular granules of the basophils. None of these characteristics has been delineated for any other fish disease described in the literature.

Beside microscopic blood findings, a sharp rise in percent. hematocrit values indicates the onset of the virus disease. However, future studies may prove that high hematocrit values are also corollary to other infections in fish. In two other instances, hematocrit values of fish have risen above the normal. Vars (1934) found that the toxicity of ammonium salts resulted in raising the mean percent. hematocrit of carp from 32 to 39. Menhaden have been exposed for various intervals of time to an asphyxiating environment by Hall, Gray & Lepkovsky (1924). The increase in hematocrit values from the normal (29.5) to 68.9 percent. was roughly proportional to the length of time of asphyxiation. These high hematocrit values of asphyxiated menhaden were attributed to the effect of the release of water from the blood to the tissues.

For the naturally infected fish, the successive number of days in which mortalities occurred ran twice as long (17 days) as it did for the experimentally infected fish (8 days). The longer duration of the disease could be the result of (1) residence in water cooler by 10° F. than that in experimentally infected fish troughs; (2) spread of the infection rather than simultaneous exposure of all fish; (3) difference in route of infection.

Acknowledgements

Physical facilities for this study were provided by the School of Fisheries, University of Washington. Sincere appreciation is accorded Dr. Q. B. DeMarsh, Department of Anatomy, Universi-

ty of Washington, for his guidance and criticism; Mr. E. F. Marten, Supervisor of Still Photography Production Unit, University of Washington, for his expert assistance with the photomicrographs; and Mr. Vladimir Lipski for his Russian translations.

Summary

Observations of cell types found in the peripheral blood of healthy and virus-diseased sockeye salmon fingerlings (*Onchorhynchus nerka*) are made with a phase-contrast microscope and reproduced photographically. Cells described include erythrocytes, thrombocytes, lymphocytes, neutrophils, eosinophils, basophils and macrophages.

Two diagnostic characteristics of the virus-diseased fish are: (1) before mortalities occur, degeneration in leukocytes and thrombocytes is made evident by the presence of an intracytoplasmic, highly refractile body which appears as a dark brown pigment granule when viewed with bright-field microscopy; (2) during the severest stage of the infection, extracellular granules of basophils appear, as well as totally degenerated leukocytes and thrombocytes which are only visible with phase-contrast microscopy.

The average percent. hematocrit of 68 control fingerlings (30-60 mm. in fork length) was 47. Hematocrit values were determined for fish which had been experimentally and naturally infected with the virus disease. At four days after experimental infection, the percent. hematocrit rose to an average of 53 (maximum 64); at 6 days, peak mortalities occurred and moribund fish registered a normal percent. hematocrit. Hematocrit values fell to a low of 16 percent. by the eighth day after exposure to the virus and then gradually rose to normal by the 27th day. Associated with below normal hematocrit values were erythematous areas at the base of the fins, at the anus or the isthmus. Microscopic blood findings and corresponding hematocrit values were related. Microhematocrit tubes used were capillary pipettes (5-10 lambda).

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EXPLANATION OF THE PLATES

All figures are photomicrographs of blood cells found in live wet films of peripheral blood which was viewed with a phase-contrast microscope.

Plate I

Fig. 1. Cell in upper right is a blebbed thrombocyte. The colorless circular area near the upper margin of this cell is a refractile body which is a pigment granule whose presence is the first evidence of cellular degeneration and denotes a virus infection.

Fig. 2. In the upper half of the field is a totally degenerated thrombocyte containing 3 pairs of pigment granules which appear as refractile bodies, each outlined by a thin dark line. In the lower half of the field is a senile erythrocyte containing a pycnotic nucleus.

Fig. 3. A non-filamented neutrophil (metamyelocyte) occupies the center and right portions of the field. Its cytoplasm contains a refractile body common in a virus infection.

Fig. 4. A normal mature erythrocyte is located left of center. Basophil normoblast is labeled *BN*. Center of the field is occupied by a totally degenerated neutrophil found in a virus-diseased fish.

Fig. 5. Macrophage which has recently ingested a large degenerated lymphocyte is characteristic of virus-diseased blood. Nucleus of macrophage is compressed at the upper right margin of the cell.

Fig. 6. Macrophage containing phagocytized granules of basophils which are numerous in a virus-diseased fish.

Plate II

Cells characteristic of blood from a virus-diseased fish.

Fig. 7. Immature basophil with only a few small, dark granules and a large nucleus.

Fig. 8. Small, round basophil filled with dark granules; nucleus is not visible.

Fig. 9. Large lymphocyte at lower right, above and to the left of which is a basophil.

Fig. 10. Degenerating basophil.

Fig. 11. Three groups of extracellular granules of basophils: upper left is a pair of granules, extreme lower right is a single granule, above which is a pair of granules unequal in size.

Fig. 12. Large leukocyte not positively identifiable but called a monocyte.

Plate III

Fig. 13. Polychromatophil normoblast between two orthochromatophil normoblasts from a virus-diseased fish.

Fig. 14. Three polychromatophil normoblasts from virus-infected blood.

Fig. 15. *L* is an active small lymphocyte with a pseudopod. A polychromatophil normoblast is to the right of the lymphocyte. All other cells are mature erythrocytes (healthy fish).

Fig. 16. Small quiescent lymphocyte (healthy fish).